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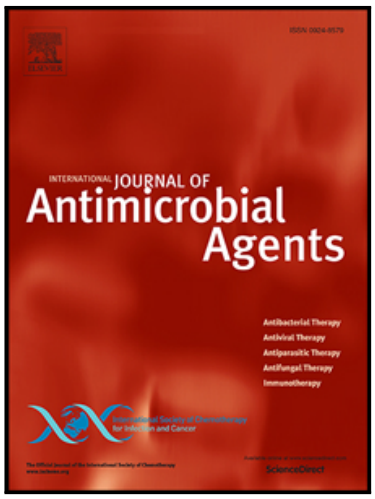

Fosfomycin Etest for Enterobacteriaceae: Interobserver and interlaboratory agreement

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Highlights

- The majority of the fosfomycin Etests shows growth of macrocolonies in the inhibition zone
- Etest observations significantly underestimate the MIC of fosfomycin, compared to the reference standard Agar Dilution
- The fosfomycin Etest has a low interobserver – interlaboratory agreement, with a higher agreement for *E. coli* compared to other Enterobacteriaceae
- Ignoring all growth in the inhibition zone might improve Etest performance

Short communication:

Fosfomycin Etest for Enterobacteriaceae: Interobserver and interlaboratory agreement.

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Abstract

Objectives: The increasing use of fosfomycin requires reliable susceptibility testing in clinical practice. The reference standard, Agar dilution (AD) is rarely used in routine settings. The fosfomycin Etest (BioMérieux) is frequently used, though the reading of MICs can be hampered by the interpretation of the growth of macrocolonies in the inhibition zone. We investigated the interobserver (IO), interlaboratory (IL), and interobserver-interlaboratory (IOIL) agreement of the fosfomycin Etest and evaluated the agreement to AD.

Methods: Etests were performed for 57 ESBL-producing Enterobacteriaceae of four bacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter cloacae*), in two laboratories. Photographs of fosfomycin Etests were interpreted by four observers following manufacturer's instructions.

Results: Essential Agreement (EA) and CA between Etest and AD was 57% and 89% (κ-value 0.68), respectively, with an underestimation of Etest interpretations compared to AD of 0.26 (95%CI: 0.03-0.48) 2-fold dilutions. Between Etest observations, IO-EA and -CA was reached in 82% and 94%; IL-EA and -CA in 38% and 85%; and IOIL-EA and -CA in 40% and 85% of comparisons, respectively. Agreement of the Etest to AD and between Etests was better for *E.coli* than for other species. Ignoring all macrocolonies and haze from Etest interpretation, improved the agreement to AD (CA κ-value 0.80) and between Etests (CA κ-value from 0.68 to 0.81).

Conclusions: In this study on 57 ESBL-producing Enterobacteriaceae, IOIL agreement was low with an EA of 40% and a CA of 85%, affected most by IL agreement and to a lesser extent by IO agreement.

Keywords:

- Etest
- Fosfomycin
- Antimicrobial susceptibility testing
- Enterobacteriaceae

Introduction:

Fosfomycin was discovered as antibiotic agent in 1969.[1] Its use has gained renewed interest due to increasing resistance against other antibiotics, especially in Enterobacteriaceae.

Fosfomycin susceptibility testing for Enterobacteriaceae is challenging in the routine setting. The reference standard, agar dilution, is complex and time consuming, making it unsuitable for routine clinical application.[2] Performance of automated broth microdilution methods is not recommended by the Clinical and Laboratory Standards Institute (CLSI) or The European Committee on Antimicrobial Susceptibility Testing (EUCAST).[3]

A potential alternative to determine the MIC of fosfomycin for Enterobacteriaceae is the Etest (BioMérieux, Durham, USA). Agreement to agar dilution varies and is described to be poor for Enterobacteriaceae other than *Escherichia coli,* attributed to difficulties in reading the Etest MIC due to growth of macrocolonies in the inhibition zone.[4,5] The manufacturer instructs to ignore up to five macrocolonies when reading the MIC.

To evaluate the Etest as an alternative fosfomycin testing method for the routine lab, we determined the interobserver (IO), interlaboratory (IL) and interobserver-interlaboratory (IOIL) essential and categorical agreement.

Materials and methods:

Isolates

Isolates originated from a collection of well-defined and sequenced ESBL-producing Enterobacteriaceae from a multicentre study on transmission in Dutch hospitals.[6 7] The selection of 57 isolates was based on the presence or absence of the FosA gene, the most frequent plasmid-borne fosfomycin resistance gene in Gram-negative bacteria, aiming at a 1:1 ratio.[8] The selection comprised 16 *Escherichia coli*, 16 *Enterobacter cloacae*, 16 *Klebsiella pneumoniae* and 9 *Klebsiella oxytoca* strains.

Microbiological procedures

Agar dilution was performed on the selection of 57 isolates, according to CLSI guidelines.[9] The bacteria were recovered from a fresh culture on a blood agar plate that was cultured overnight at 35-37°C. Next, a suspension of 0.5 McFarland of bacteria in 0.45% NaCl (10^8 CFU/mL) was made and diluted to 10^7 CFU/mL in 0.9% NaCl. Bacterial suspensions were pipetted per 12 into a 24- wells plate and replicated. Subsequently, 2 µL bacterial inoculum (±1x10^4 CFU/spot of 5-8 mm) from each well was inoculated onto a Mueller-Hinton II agar plate, containing 25 mg/L glucose-6-phosphate and fosfomycin in concentrations from 0.25 mg/L to 128 mg/L. The agar plates were incubated for 16-20 hours at 35-37°C. The highest fosfomycin concentration, at which no visible bacterial growth on the agar plate was observed by the naked eye, was considered the MIC. Single colonies or a weak haze due to the bacterial inoculum were ignored.

Etest susceptibility testing was performed according to the manufacturer's instructions in two Dutch clinical microbiology laboratories. A suspension of 0.5 McFarland of overnight cultured bacteria in 0.85%NaCl was inoculated onto a Mueller-Hinton II agar (MHA: Oxoid in laboratory A and Becton Dickinson in laboratory B). In both laboratories a sterile swab was used, streaking the agar surface three times rotating the plate 60 degrees each time. Within 15 minutes after inoculation, Etest strips were applied onto the inoculated MHA. Plates were incubated for 16-20 hours at 35-37 ºC. Photographs of the incubated agar plates were made to enable independent reading of inhibition zones (example in supplementary material).

Etest interpretation

Four clinical microbiology residents interpreted all photographs independently, resulting in 8 separate Etest observations for 57 bacterial isolates. First, observers were instructed to register the number of macrocolonies present in the inhibition ellipse; second, to ignore all macrocolonies and haze to determine the MIC at 80% inhibition ($MIC_{80\%}$); and third, to include all macrocolonies to determine the MIC at 100% inhibition ($MIC_{100\%}$). According to manufacturer's instructions, the MIC used for the main analysis (recommended MIC) was $MIC_{80\%}$ if five or less macrocolonies were observed and $MIC_{100\%}$ in case of more than five macrocolonies.

Outcome measurement

Essential agreement (EA) was defined as agreement of Etest MIC values within one MIC dilution step, and Categorical agreement (CA) as MIC values within the same EUCAST susceptibility category, i.e. susceptible (MIC ≤32 mg/L) or resistant (MIC >32 mg/L). [2]
Agreement was calculated between Etest and AD, and between the following combination of

Etest observations. Interobserver (IO) agreement was defined as agreement between individual observers within one laboratory; interlaboratory (IL) agreement as agreement between the observations of the same observer for Etests performed in the two laboratories and interobserver-interlaboratory (IOIL) agreement as agreement between combinations of different observers and different laboratories, best reflecting clinical practice. Disagreements were classified as very major errors (VME) if the Etest resulted in a susceptible and AD in a resistant result and major errors (ME) if the Etest resulted in a resistant and Agar Dilution in a susceptible result.

Statistical analysis

Cohen's kappa test was used to evaluate CA, as it accounts for the possibility of CA occurring by chance. The κ results is a value between 0, which represents no agreement, and 1, representing complete agreement [10]. We also determined the systematic difference between AD and Etest and between Etest observations in laboratory A and laboratory B by calculating the mean difference in 2 fold dilution steps.

IBM SPSS Statistics (version 21) was used for statistical analyses.

Results:

Etest interpretation

Due to low quality of the photographs, 2/456 Etest observations were not interpreted, leaving 454 Etests for analysis. Growth of macrocolonies within the inhibition zone was reported in 268 of 454 (59%) Etest interpretations, (laboratory A 132/228 (58%); laboratory B 136/226 (60%)). In 71 of 454 observations (16%) 5 or more macrocolonies were observed, meaning a switch in the recommended MIC from $MIC_{80\%}$ to $MIC_{100\%}$.

Agreement Etest to AD

Overall, EA and CA between the Etest MIC and AD was 57% and 89%, respectively (mean κ-value 0.68, 95%CI 0.42:0.95, table 1). Categorical disagreement resulted in 4% VMEs and 7% MEs. Small differences existed between laboratories and observers. For *E. coli*, CA between Etest and AD was 100%, in contrast with the other species (range 77-91%). Reading the MIC at 80% inhibition resulted in a higher agreement than the recommended MIC (mean κ-value 0.80, 95%CI 0.54:1.07). We observed a significant systematic difference between mean AD and Etest of 0.26 (95%CI: 0.03:0.48) 2-fold dilutions.

Agreement between Etest observations

The overall EA between all Etest observations was 911/1582 (58%) and CA was 1404/1582 (89%) with a κ-value of 0.68 (95%CI:0.63:0.73, table 2). The IO agreement was higher than the IL-agreement. The $MIC_{80\%}$ interpretation resulted in a significantly higher kappa (0.81, 95%CI 0.76-0.86) than the recommended MIC. EA and CA between all eight observations was reached for 8/57 (14%) and 37/57 isolates (65%), respectively. CA was 100% for *E.coli,* and lower for the other species. EA was highest for *E.cloacae* (70%). The mean systematic difference between Etest observations in laboratory A and laboratory B was 1.60 (95%CI: 1.32:1.88) in 2 fold dilution steps.

Discussion:

In this study on 57 ESBL-producing Enterobacteriaceae strains of four different species, IOIL agreement was low (EA 40%, CA 85%), affected most by IL agreement and to a lesser extent by IO agreement. No previous studies reported the IO or IL agreement for reading the fosfomycin Etest. A systematic difference was found with significantly higher MIC's observed in laboratory A than in laboratory B.

Factors that may have affected IL agreement were the materials used – such as the Mueller Hinton agar (a non-synthetic medium that may differ in composition between companies) - and the technician that performed the test. It confirms that there is a significant variation in MIC determination between labs, and MIC values obtained should be regarded with certain caution.[11]

A significant systematic difference was found between AD and Etest leading to an underestimation of the Etest. The low agreement of Etest observations to AD is in line with other studies, as well as the higher agreement for *E. coli* compared to other Enterobacteriaceae.[5,12,13] In contrast to the other species, in *E. coli* the presence or absence of the FosA gene resulted in either very high or very low MICs.

Growth of macrocolonies in the inhibition zone was observed in the majority of Etests. Ignoring macrocolonies and haze from interpretation ($MIC_{80\%}$) improved CA to AD (from 89% to 94%, mean κ-value 0.80) and between Etest observations (from 89% to 95%, κ-value 0.81). Our results suggest that the more feasible $MIC_{80\%}$ interpretation performs better than the recommended MIC; this observation should be confirmed in larger cohorts.

Our study has several limitations. Firstly, we used a small population of ESBL-producing Enterobacteriaceae isolates from hospitalized patients in the Netherlands. The majority of isolates appeared susceptible to fosfomycin using the current breakpoints. This could affect the generalizability of the results. Otherwise, we aimed to include a large enough number of resistant strains to allow a good estimate of VMEs, as this is can be a problem when using isolates with from large surveys with a low resistance frequency. Secondly, we did not interpret the actual Etests, but the photographs, which is not the normal practice.

Conclusions:

In conclusion, the fosfomycin Etest has a low IO-IL agreement and low agreement to AD. The observed variations in the interpretation of the fosfomycin Etest questions its general use in clinical practice. The better performance for *E.coli* isolates compared to other species supports the suggestion to limit its use to *E.coli*. [4,5] Finally, performance and feasibility might improve when ignoring all growth in the inhibition zone.

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Declarations

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Competing Interests: All authors report no conflicts of interest relevant to this article.

Ethical Approval: Ethical approval was obtained for the SoM study. The SoM study was reviewed by the Medical Research and Ethics Committees of the Elisabeth-TweeSteden Hospital (Tilburg, the Netherlands). The study was judged to be beyond the scope of the

Medical Research Involving Human Subjects Act (WMO), and a waiver of written informed consent was granted (SoM: METC/jv/2010.034; R-GNOSIS: WAG/om/13/069083).

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Table 1: Comparison between fosfomycin Etest and agar dilution

							Very major error (%))*	**Major error (%))****
Agar dilution		57	11 (19)	N/A	N/A	N/A	N/A	N/A
Etest - recommended MIC	Total	454	102 (22)	261 (57)	0.68 (0.42-0.95)	406 (89)	17 (4)	31 (7)
	Laboratory A	228	41 (18)	100 (44)	0.70 (0.44-0.97)	207 (91)	12 (5)	9 (4)
	Laboratory B	226	61 (27)	161 (71)	0.67 (0.40-0.93)	199 (88)	5 (2)	22 (10)
	Observer 1	114	26 (23)	68 (60)	0.68 (0.42-0.95)	102 (89)	4 (4)	8 (7)
	Observer 2	114	22 (19)	58 (51)	0.73 (0.46-0.99)	104 (91)	5 (4)	5 (4)
	Observer 3	114	25 (22)	65 (57)	0.71 (0.44-0.97)	103 (90)	4 (4)	7 (6)
	Observer 4[##]	112	29 (26)	70 (63)	0.62 (0.36-0.89)	97 (87)	4 (4)	11 (10)
	E.coli	128	64 (50)	80 (63)	N/A[$]	128 (100)	0 (0)	0 (0)
	K. pneumoniae	128	19 (15)	57 (45)	N/A[$]	99 (77)	13 (10)	16 (13)
	K. oxytoca	71	8 (11)	35 (49)	N/A[$]	63 (89)	4 (6)	4 (6)
	E. cloacae	127	11 (9)	89 (70)	N/A[$]	116 (91)	0 (0)	11 (9)
Etest - $MIC_{80\%}$		454	81	289	0.80 (0.54-	427	17 (4)	10 (2)

		(18)	(64)	1.07)	(94)		
Etest - $MIC_{100\%}$[###]	453	225 (50)	221 (49)	0.23 (-0.03-0.50)	294 (65)	11 (2)	148 (33)
CLSI breakpoint for Etest and AD (S>64 mg/L)	454	88 (19)	261 (57)	0.62 (0.36-0.89)	402 (89)	22 (5)	30 (7)

* Etest gives a susceptible result and Agar Dilution a resistant result.

** Etest gives a resistant result and Agar Dilution a susceptible result.

[#]Agar Dilution measured MIC's up to ≥128mh/L. For these isolates Etest MIC's ≥64mg/L were classified as Agreement

[##]Observer 4 rated two Etests as not assessable because of low quality of the photographs; these were excluded from all analyses.

[###]Observer 4 left one MIC100 result empty.

[$]Number of isolates for individual species were too low to calculate reliable Kappa values.

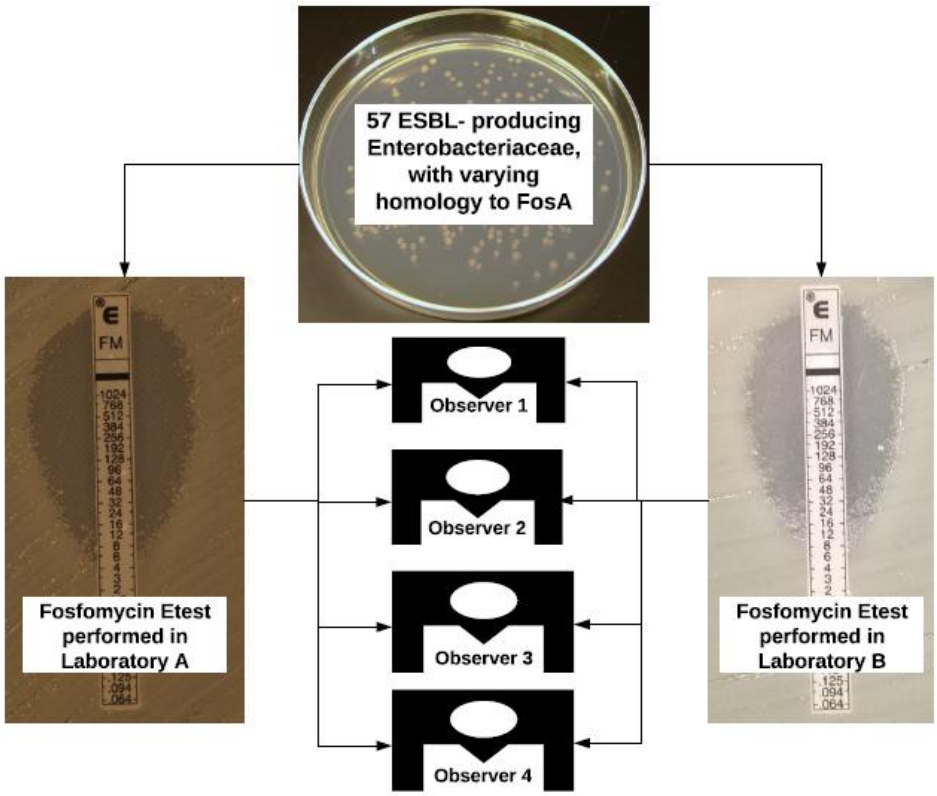
Table 2: Overall, interobserver, interlaboratory and interlaboratory-interobserver agreement for the reading of fosfomycin Etest, provided by the kappa value, categorical agreement (CA,%) and essential agreement (EA,%)

Variable		**N of comparisons**	**Essential Agreement (%)**	**Categorical Agreement (%)**	**Kappa (95%CI)**
Recommended MIC		1582	911 (58)	1404 (89)	0.68 (0.63-0.73)
Interobserver	Laboratory A	342	258 (75)	321 (94)	0.79 (0.68-0.90)
	Laboratory B	336	297 (88)	315 (94)	0.84 (0.74-0.95)
Interlaboratory	Observer 1	57	19 (33)	49 (86)	0.60 (0.34-0.87)
	Observer 2	57	18 (32)	51 (89)	0.66 (0.40-0.93)
	Observer 3	57	22 (39)	48 (84)	0.54 (0.27-0.80)
	Observer 4[#]	55	26 (47)	44 (80)	0.50 (0.23-0.76)
Interobserver-interlaboratory		678	271 (40)	576 (85)	0.58 (0.28-0.84)[*]
E.coli		448	287 (64)	448 (100)	1.00 (0.91-1.09)
K. pneumoniae		448	186 (42)	359 (80)	0.21 (0.12-0.31)
K. oxytoca		245	129 (53)	213 (87)	0.36 (0.24-0.49)
E. cloacae		441	309 (70)	384 (87)	0.20 (0.10-0.29)
$MIC_{80\%}$		1582	977 (62)	1495 (95)	0.81 (0.76-0.86)
$MIC_{100\%}$[##]		1575	900 (57)	1240 (79)	0.58 (0.53-0.63)
CLSI breakpoint		1582	911 (58)	1450 (92)	0.74 (0.69-0.79)

[#] Observer 4 rated two Etests as not assessable because of low quality of the photographs; these were excluded from all analyses.

[##]Observer 4 left one MIC100 result empty.

[*]Mean kappa with mean 95% confidence interval



Graphical abstract: The inter-observer and inter-laboratory agreement of the fosfomycin E-test in ESBL-producing Enterobacteriaceae